EXAMINATION OF SOME SOLUBLE CONSTITUENTS OF SPHAGNUM GAMETOPHYTES[1]

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Abstract

A survey of 14 species of *Sphagnum* has shown that the amino acids and organic acids are essentially those encountered in higher plants. At least one unidentified amino compound occurs in the rare *S. strictum*. Malic and citric acid are the dominant organic acids, but the amounts of free organic acids in *Sphagnum* are unusually low. Two tri- and three tetra-saccharides, consisting of fructosylated sucrose, are regular constituents of the neutral fraction of *Sphagnum*.

Introduction

One is surprised to note how little information is available on the chemical constituents of bryophytes in contrast to data on higher plants. Even a recent standard work on plant constituents (Hegnauer 1962), indicates that the non-volatile acids of the tricarboxylic acid cycle are absent in bryophytes (Allsopp 1951). The Sphagnobrya or peat mosses, particularly, have never been studied thoroughly. For instance, amino acids have only been examined in one species (Black *et al.* 1955). More information on their biochemistry may help us to understand how they manage to survive such extreme habitats as peat bogs.

A survey of the main soluble constituents of a number of *Sphagnum* species was carried out to narrow this information gap. The Maritime Provinces of eastern Canada are especially rich in interesting and rare species such as *S. macrophyllum*, *S. pylaesii*, etc., and provide an excellent area for comparative studies on the genus. With the 14 species selected, the subdivisions of the genus are taxonomically well represented, and species from various habitats have been included.

Materials and Methods

Chemical analysis was performed on the 14 species listed in Table I. Fifty to one hundred grams fresh weight of the apical regions (cut a few millimeters below the heads) were carefully sorted and washed. Surface water was removed by blotting and portions of the plant material were placed in a force draft oven at 100 °C for dry weight estimations.

The materials to be analyzed were ground to a flour in liquid nitrogen with a mortar and pestle and subsequently fractionated using the ion-exchange technique of Bové *et al.* (1957). This flour was added to ice water containing Permutit Q resin and the pH of the mixture adjusted to one with HCl. The resin was removed by decantation and the precipitated proteins together with the insoluble cell wall material were removed by centrifugation. The amino acids were recovered from the resin. The supernatant was fractionated on Dowex 2 into an organic acid fraction and a neutral fraction. *S. compactum* was examined only for amino acids, and an air dry herbarium specimen of *S.*

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TABLE I

Collection data of *Sphagnum* species, arranged in taxonomical order. With the exception of *S. aongstroemii*, the materials were harvested in late November and early December, 1961

Section	Species	Habitat and distribution
Palustria	*S. magellanicum* Brid.	Bog, dry hummocks, exposed, widely distributed
Palustria	*S. papillosum* Lindb.	Poor fen, periodically inundated, exposed, widely distributed
Acutifolia	*S. angermanicum* Melin emend. Maass	Poor fen, periodically inundated, suboceanic species with sparse representation in Europe
Acutifolia	*S. flavicomans* (Card.) Warnst.	Poor fen, dry hummocks, exposed, suboceanic endemic species
Polyclada	*S. wulfianum* Girg.	Spruce–cedar swamp, hummocks on raw humus, shaded, continental species
Rigida	*S. strictum* Sull.	On humic claysand in wet depressions of *Myrica–Chamaedaphne* heather, shaded, oceanic species
Rigida	*S. compactum* DC.	In depressions of boggy heather, exposed, widely distributed
Squarrosa	*S. teres* (Schimp.) Aongstr.	Rich fen, hummocks, exposed, widely distributed
Cuspidata	*S. tenellum* Pers.	Poor fen, inundated, exposed, suboceanic species
Cuspidata	*S. torreyanum* Sull.	Mesotrophic shallow pond, submerged, exposed, suboceanic endemic species (doubtful in Europe)
Cuspidata	*S. macrophyllum* Bernh. s. str.	Mesotrophic shallow pond, submerged and shaded by a coating of muddy particles, oceanic endemic species
Subsecunda	*S. subsecundum* Nees s. lat.	Wet and shaded rocks, forming carpets, oceanic variety of a widely distributed species
Subsecunda	*S. pylaesii* Brid.	Mesotrophic shallow pond, submerged, exposed, oceanic species with sparse representation in Europe
Truncata	*S. aongstroemii* Hartm.	Herbarium specimen from Finland collected by Brotherus in 1912 near Ruovesi (duplicate material), a generous gift from the herbarium of the University of Helsinki

aongstroemii only for sugars. For the isolation of oligosaccharides from *S. teres*, the plant material was extracted with boiling 50% ethanol. The extract was deionized after removal of the ethanol.

Amino Acids

These were identified by two-directional paper chromatography using (1) phenol:water (3:1) plus 0.8% 8-hydroxyquinoline, adjusted to pH 5.4 with NaOH, for the first direction and (2) *n*-butanol:acetic acid:water (9:1:4) for the second. Spots were identified with (*a*) ninhydrin, (*b*) isatin, (*c*) Sakaguchi reagent for guanido compounds, (*d*) Ehrlich's reagent for citrulline, (*e*) vanillin reagent for ornithine and proline, and (*f*) sulphanilic acid reagent for histidine (Block *et al.* 1955).

Organic Acids

The ammonium carbonate eluate from Dowex 2 was acidified and continuously extracted with ether for 6 hours. The organic acids thus obtained were fractionated on silicic acid columns according to the method of Bové *et al.*

(1957). This was done for five species. In the case of the other species one-directional chromatograms were made using (1) 95% ethanol:ammonium hydroxide:water (84:5:11), (2) *n*-propanol:methylbenzoate:90% formic acid:water (28:12:8:10), and (3) ether:formic acid:water (5:2:1). Organic acids were detected by acid/base indicator sprays and specific identifications were made by the method of Martin (1955).

Sugars

The neutral fraction was chromatographed for sugars on Whatman 3 MM paper using the following solvents: (A) *n*-butanol:95% ethanol:water (525:320:155), (B) *n*-propanol:ethylacetate:water (7:2:1), (C) *n*-butanol:pyridine:water (6:4:3), (D) isopropanol:pyridine:water:acetic acid (8:8:4:1), and (E) ethylacetate:pyridine:water (8:2:1). A combination of the aniline hydrogen phthalate spray reagent followed by 2% orcinol in 2 *N* HCl was usually used to detect the sugars. Reagents such as ammoniacal silver nitrate and periodate–benzidine failed to reveal additional spots on test chromatograms of the neutral fractions. Direct chromatography of 50% ethanol extracts gave the same results as the ion exchange procedure, therefore, the formation of artifacts resulting from partial hydrolysis was considered unlikely. Oligosaccharide spots were eluted from chromatograms developed in solvent (A) and hydrolyzed either with 2 *N* formic acid at 108 °C for 1 hour or with yeast invertase (0.01 *N* acetate buffer, pH 4.6 at 22 °C) to determine the constituent monoses. These were separated by solvent (E). Preparative column chromatography on charcoal:celite (1:1) was found to be unsatisfactory for the isolation of the oligosaccharides (see results and discussion). The oligosaccharides of *S. teres* were fractionated on cellulose powder (Hough *et al.* 1949) using a butanol:95% ethanol:water (525:320:50) mixture whose water content was gradually increased. This was followed by preparative paper chromatography in solvent (A) on washed Whatman 3 MM sheets. The oligosaccharides thus obtained were hydrolyzed according to the AOAC methods for sucrose and the molar ratios of the constituent monoses (fructose and glucose) were determined after paper chromatographic separation in solvent E using the anthrone technique of Yemm and Willis (1954).

TABLE II

Yields of neutral, amino acid, and organic acid fractions of 12 species of *Sphagnum*

Group	Species	Amino acids, % of dry wt.	Neutral fr., % of dry wt.	Organic acids, μeq/g of dry wt.
Palustria	*S. magellanicum*	0.16	8.8	16.7
	S. papillosum	0.08	4.5	10.0
Acutifolia	*S. angermanicum*	0.41	15.5	23.2
	S. flavicomans	0.16	5.9	9.1
Polyclada	*S. wulfianum*	0.14	4.4	6.7
Rigida	*S. strictum*	0.56	8.4	32.4
Squarrosa	*S. teres*	0.17	19.0	82.5
Cuspidata	*S. tenellum*	0.25	6.3	29.7
	S. torreyanum	0.50	13.9	19.8
	S. macrophyllum	0.96	2.9	15.2
Subsecunda	*S. subsecundum*	0.16	10.0	12.5
	S. pylaesii	1.34	5.1	8.5

Results and Discussion

Amino Acid Fraction

The total amount of soluble amino compounds varied from about 0.1% to 1.3% of the dry weight (Table II). The lowest yields were from species exhibiting hummock type of growth (*S. magellanicum*, *S. papillosum*, *S. flavicomans*, *S. wulfianum*, *S. teres*) and the highest from aquatic species (*S. pylaesii*, *S. macrophyllum*).

The distribution of soluble amino acids in 12 species of *Sphagnum* harvested at the end of the growing season is shown in Table III. The dominant amino acids regularly found were aspartic and glutamic acids and their amides, as well as α-alanine and serine. With the exception of tyrosine, all the free amino acids listed by Black *et al.* (1955) for *S. imbricatum* (a species belonging to the Palustria group) were observed. In addition, phenylalanine, citrulline, and ornithine were detected in a number of species examined here. Of the unidentified amino compounds, A_1 ran as fast as proline in the phenol solvent (R_f value 0.9), but in the butanol solvent hardly at all (R_f value 0.05). It gave an unusual slate-grey color with ninhydrin and a red color with isatin. A_2 moved very slowly in both solvent systems and was present only in trace amounts. There was some variation both in the occurrence and the amounts of certain amino acids, especially the basic amino acids. These differences neither reflected the division of the genus into taxonomically founded groups nor differences in the habitats such as water or light conditions (compare Table I). Seasonal changes may be more important for some of the variation observed. For instance, A_1 was the dominant compound in the amino acid fraction of *S. strictum* collected between June and December. In contrast, material collected between January and April contained very little of this compound. Because of the limited amount of material available, its isolation was not attempted. *S. compactum*, a close relative of *S. strictum* and a much more common species, did not yield the compound.

Organic Acids

The total amount of organic acids present in *Sphagnum* was low as compared with higher plants and varied from approximately 7 to 82 microequivalents per gram of dry weight (Table II). Both the highest and lowest values were found in species from hummocks (*S. teres*, *S. wulfianum*). Although the organic acids of *Sphagnum* have been implicated in bog ecology (Ramaut 1955), their quantities have not been determined previously. In the only study known to us, Ramaut (1955) isolated a single organic acid from *S. recurvum* (a member of the Cuspidatum group) which appeared to be either succinic acid or a polymer of it.

A typical elution curve of organic acids from *Sphagnum* separated on a silicic acid column is shown in Fig. 1. Quantitative estimations of the organic acid peaks from the five species examined are given in Table IV. Fumaric, succinic, oxalic, malic, and citric acids were identified by paper chromatography as well as by their elution volumes. The two major acids were malic and citric acids. In some species oxalic acid was present in significant amounts. Contrary to Ramaut's finding with *S. recurvum*, succinic acid was found to be only a minor constituent. Essentially the same pattern was given by the rest of the

TABLE III

Distribution of soluble ninhydrin-positive substances in 12 species of *Sphagnum*, representing the major groups of the genus and harvested at the end of the growing season. The ninhydrin reactions were evaluated as follows: + faint, ++ moderate, +++ strong. The amount of extract chromatographed was 0.5 mg in each case

Group	Species	Ala	Phe	γ-Amino butyric	Arg	Asp	Asp.NH_2	Citrulline	Glu	Glu.NH_2	Gly
Pal	*S. magellan.*	++		+		++	+		+++		+
	S. papillos.	++		+		++	++		+++	+++	
Acu	*S. angerman.*	++	+	+	++	+++	++	+	+++	+++	
	S. flavicom.	++	++	++		+++	++	+	+++	+++	+
Pol	*S. wulfianum*	+++	+	++	+	++	++	+	+++	+++	+
Rig	*S. strictum*	++	+		+	++	++		+++	++	
Squ	*S. teres*	+++		++	+	+++	+		+++	++	+
Cus	*S. tenellum*	++		+		++	++		+++	++	+
	S. torreyan.	++		+		+++	+++	+	+++	+++	
	S. macroph.	++	+	+	++	+++	++	++	+++	+++	
Sub	*S. subsec.*	++		+		++			+++		+
	S. pylaesii	+	+	+	++	+++	++	+	+++	+++	

Group	Species	His	Leu	Lys	Met	Orn	Pro	Ser	Thr	Val	A_1	A_2
Pal	*S. magellan.*		++					++	+	+		+
	S. papillos.	+	+					++	+	+		
Acu	*S. angerman.*	+				+		++	+			+
	S. flavicom.	+	++			+		+++	+			+
Pol	*S. wulfianum*	+		++		++		+++	+			
Rig	*S. strictum*	++	+	+		+	++	+++	+++		+++	
Squ	*S. teres*	+	++	+			+	++	+	+		+
Cus	*S. tenellum*	++	++		+			++	+			+
	S. torreyan.	+	+				+	++	+	+		
	S. macroph.	++	++			+	+	+	+			
Sub	*S. subsec.*		++	+			+	++	+	+		
	S. pylaesii	++	+			+	+	+	+	+		

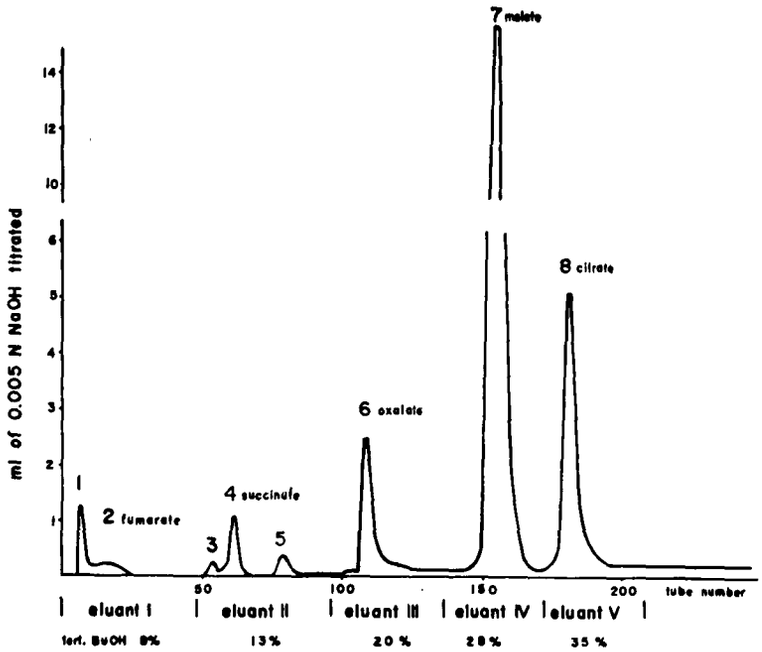

FIG. 1. Separation of the organic acid fraction of *Sphagnum teres* on a silicic acid column, according to the procedure of Bové *et al.* (1957). Elution with increasing amounts of tert. butanol in chloroform saturated with 0.5 *N* sulphuric acid.

species, as revealed by two-directional paper chromatography. This distribution of organic acids is similar to that generally found in higher plants, whereas in liverworts only aconitic and malic acids have been observed (Das and Rao 1963). No work appears to have been done on the organic acids of true mosses (Musci).

Neutral Fraction

The yield of the neutral fraction was the highest of all soluble fractions and varied from about 3% of the dry weight in an aquatic species (*S. macrophyllum*) to 19% of the dry weight in a terrestrial species (*S. teres*).

TABLE IV

Organic acid fractions from five species of *Sphagnum*, obtained by column chromatography on silicic acid. The yields are expressed as a percentage of the total organic acids as determined by titration

Peaks eluted	Org. acids identified	*papillosum*	*strictum*	*teres*	*torreyanum*	*macrophyllum*
1	?	5.1	2.2	2.0	4.2	5.7
2	Fumaric	—	1.2	Trace	2.1	4.2
3	?	Trace ?	4.1	0.4	1.2	3.8
4	Succinic	8.2	3.0	2.4	9.7	3.8
5	?	—	1.2	0.9	Trace ?	Trace ?
6	Oxalic	9.4	2.0	8.0	16.4	Trace ?
7	Malic	64.0	66.7	65.2	47.2	67.5
8	Citric	13.6	19.6	21.2	19.1	15.0

TABLE V

$R_{sucrose}$ values of *Sphagnum* fructosides (U_1 to U_7) and of melizitose, raffinose, and stachyose standards, as compared by descending paper chromatography on Whatman 3 MM paper at room temperature. Average from three determinations (maximum deviation ± 0.03)

	Solvent system*			
$R_{sucrose}$ values	A	B	C	D
Fructose	2.50	1.70	1.35	1.09
Sucrose	1.00	1.00	1.00	1.00
Melizitose	0.49	0.48	0.68	0.86
Raffinose	0.30	0.32	0.49	0.69
Stachyose	0.08	0.07	0.19	0.37
U_1	0.59	0.54	0.74	0.90 (0.86)†
U_2	0.49	0.54	0.73	0.89 (0.85)†
U_3	0.27	0.31	0.54	0.87 (0.80)†
U_4	0.17	0.20	0.42	0.85 (0.75)†
U_5	0.10	0.12	0.30	0.83 (0.69)†
U_6	0.00	0.00	0.18	— ‡
U_7	0.00	0.00	0.00	0.00§

*See Materials and Methods.
†As a result of acid lability, no discrete spots were formed, and a streak was observed which reached the area of fructose. Although in these cases, too, the center of color reaction was taken as an $R_{sucrose}$ value, it was more meaningful to determine the position of the back end of the spots as well (in parentheses).
‡Not determined.
§A considerable portion of the fructan was hydrolyzed within 24 hours, yielding a strong streak fading towards fructose.

The soluble carbohydrates of *Sphagnum* were found to consist of glucose, fructose, sucrose, and a series of fructosides. Sucrose was crystallized from *S. palustre* by Goris and Vischniac (1913), and from *S. balticum* by Theander (1954). The presence of fructosides was indicated by paper chromatography (Theander 1954; Black *et al.* 1955; Chollet and Dufour 1955; Quillet *et al.* 1956). According to Chollet and Dufour (1955), these fructosides accounted for 90% of the soluble carbohydrates in *S. palustre*. Although Chollet and Dufour (1955) did not positively identify raffinose and stachyose, these sugars have been listed as constituents of *Sphagnum* (Karrer 1958; Hegnauer 1962).

Our chromatographic studies have shown that at least six unknown fructosides can be found in the region between sucrose and the origin (see Table V). The fructosides were demonstrated in protonemata as well as in gametophytes. None of them corresponded to raffinose or stachyose. Since *S. aongstroemii* also contained the fructosides, all the taxonomical groups of the genus *Sphagnum* appear to be chemically similar in this respect. We have observed an identical series of sugars in the liverwort, *Scapania undulata*. These have been compared with the fructosides of *Helianthus tuberosus*, *Dahlia variabilis*, and *Phleum pratense* var. *bulbosum* and appear to be different.

The unidentified fructosides were assigned symbols (U_1 to U_7) and their migration rates relative to sucrose are presented in Table V. U_1 and U_2 were only separable in solvent A, and U_6 migrated only in solvent C.

Acid hydrolysis of U_1 to U_5 yielded only fructose and glucose. The material remaining on the origin of chromatograms developed in solvent A (U_6 and U_7) contained galactose and trace amounts of mannose, arabinose, xylose, rhamnose, and fucose, in addition to glucose and fructose. Enzymic hydrolysis by yeast invertase was effective in U_1 to U_5, again yielding only fructose and

TABLE VI

Ratios of fructose:glucose found after hydrolysis of the unknown oligosaccharides from the neutral fraction of *Sphagnum*

Oligosaccharide	Fructose/glucose	Molar ratio
U_1	1.95	2:1
U_2	1.75	2:1
U_3	2.79	3:1
U_4	3.15	3:1
U_5	2.78	3:1

glucose. U_1 was hydrolyzed almost as rapidly as sucrose, while U_2 to U_5 were hydrolyzed at progressively lower rates. No invertase action was detectable after 1 hour in the case of U_6 and U_7.

The molar ratios of the hydrolysis products of the fructosides are presented in Table VI. The results indicate the presence of two trisaccharides (U_1 and U_2) and three tetrasaccharides (U_3, U_4, and U_5) in *Sphagnum*. The hydrolysis of U_6 was not attempted as it was only a minor compound and centered in a streak possibly containing heterogenous materials. Likewise the origin (U_7) probably consists of a mixture of polysaccharides, including a fructan. Hegnauer (1962) refers to the fructan of *Sphagnum* as an "inulin-like polyfructoside reserve" which is similar to synanthrin from *Helianthus tuberosus* in molecular size and other properties (cf. Chollet and Dufour 1955). Our results do not support the assumption (Hegnauer 1962; cf. Chollet and Dufour 1955) that the soluble fructosides in *Sphagnum* are simply a linear series of increasing molecular weight. The reactivity of these compounds with invertase strongly indicates that these are fructosyl-sucrose sugars as suggested by Hegnauer (1962). Moreover, it was observed that a colorless preparation of U_1 plus U_2, in concentrated aqueous solution and stored in a deepfreeze for a year, hydrolyzed slowly to give sucrose and fructose. One additional day at room temperature caused U_1 to disappear completely, leaving a little U_2 and fructose plus sucrose. No microbial action was detected during this change. While the (acid) lability especially of U_1 is striking, it is surprising that the complete series of fructosides (U_1 to U_5) could be identified in an extract from a 50-year-old herbarium specimen of *S. aongstroemii*. In contrast, Black *et al.* (1955) were not even able to detect fructose in an air-dried sample of *S. imbricatum*.

Usually, there was less of the trisaccharide U_1 than of U_2, even when the mildest extraction procedure was used. Of the tetrasaccharides, U_5 was regularly dominant. A pure culture of the protonema of *Sphagnum inundatum* yielded U_5 as the strongest spot together with the fructan, while sucrose and fructose were not detected at all.

While the trisaccharides were not found to be separable on a charcoal column, the tetrasaccharides were partly resolved, U_3 forming a single peak behind the trisaccharide peak, and U_4 and U_5 forming a double peak. Although the elution pattern appeared to be encouraging, the sugars were contaminated by smaller amounts of materials from the neighboring peaks. The recovery of the trisaccharides from the trisaccharide peak was only about 30%. Similar diffi-

culties with charcoal chromatography have been encountered in the separation of glucofructosides from higher plants (Bacon 1959).

In *Helianthus annuus*, the sugars following sucrose on paper chromatograms were found to be a tri- and a tetra-saccharide (Dedonder 1952). An extract from *Helianthus* tubers gave an almost matching series of spots when compared with *Sphagnum* extracts after paper chromatography in solvent A. In solvent C however, the spots appeared to be very different.

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